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THE ROLE OF INTERLEUKIN-6 IN LIPOPOLYSACCHARIDE-INDUCED WEIGHT LOSS, HYPOGLYCEMIA AND FIBRINOGEN PRODUCTION, IN VIVO

Gideon Strassmann, Miranda Fong, Sandra Windsor, Ruth Neta*

It was recently shown that interleukin (IL)-6 is an important mediator involved in the Colon (C)-26 model of experimental cancer cachexia. In this study, we wished to determine whether IL-6 is also involved in several metabolic changes associated with lipopolysaccharide (LPS) challenge. Administration of a relatively high amount of LPS to mice induced a transient weight loss, hypoglycemia, hypertriglyceridemia and an increase in the hepatic acute phase reactant, fibrinogen. Pretreatment of mice with the rat anti-murine IL-6 antibody (20F3), but not with a control antibody, resulted in a significant improvement of LPS-induced hypoglycemia and weight loss as well as a significant decrease of plasma fibrinogen. Anti- IL-6 antibody had no effect on LPS-induced hypertriglyceridemia. On the other hand, the pretreatment of mice with anti-murine TNF (TN3.19) antibody was able to completely inhibit elevation of triglycerides and modestly improve LPS-induced weight loss although it had no effect on hypoglycemia and fibrinogen production. Taken together, these results suggest that IL-6 plays a role in some of the metabolic changes associated with both an acute (i.e. LPS challenge) and chronic (C-26 cachexia) inflammatory conditions.

Prolonged exposure to an inflammatory stimulus may result in chronic wasting or cachexia. In neoplastic diseases, the presence of wasting of muscle and fat tissues is quite frequent[1] and complicates therapeutic intervention.[2] Tumor necrosis factor (TNF) has been suggested as a mediator of cancer cachexia because it suppresses metabolic enzymes such as lipoprotein lipase (LPL) activity and induces anorexia and weight loss when administered into experimental animals.[3-5] Recently, however, an experimental cachexia model has been identified that appears to involve another factor. This model involves a cell line derived from colon (C)-26 adenocarcinoma, which retains the transplantability of the original tumor and causes severe weight loss in syngeneic hosts.[6] In this model, we showed that interleukin (IL)-6 appears to have a more significant role than TNF in mediating the various parameters of wasting. This was based on the finding that the monoclonal antibody (MAb) against murine IL-6 (20F3) but not a MAb against murine TNF (TN3.19) could prevent the development of key parameters of cachexia, including weight loss, hypoglycemia and hepatic acute phase response.[6] More recently, IL-6 was also found to reduce LPL activity in vitro and in vivo.[7]

Profound metabolic disturbances are not restricted to chronic diseases but can also occur during infection.[8] Many of these metabolic changes can be reproduced by the administration of bacterial products such as lipopolysaccharides (LPS).[9,10] LPS induces TNF, IL-1 and IL-6, each of which is thought of as primary mediator of acute inflammation, since direct administration of each of them mimics some inflammatory events. The overlapping effects of cytokines, together with their induction of each other, as in the case of IL-1 and TNF, as well as the induction of IL-6 by both IL-1 and TNF, has complicated the precise determination of their individual contributions to events associated with acute inflammation.

From the Department of Immunology, Otuska America Pharmaceutical, Inc., Rockville, MD 20850, and *Department of Experimental Hematology, Armed Forces Radiobiology Research Institute, Bethesda, MD 20814, USA.
Address correspondence to: Dr G. Strassmann, Otsuka America Pharmaceutical, Inc., 9900 Medical Center Drive, Rockville, MD 20850, USA.
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In this report, using neutralizing anti-cytokine MAb, we attempted to determine the relative contribution of TNF and IL-6 in several metabolic changes associated with endotoxin shock in vivo.

RESULTS

LPS Induces Transient Weight Loss

To determine whether LPS induces weight loss in CD_2F_1 mice, increasing amounts of LPS were injected and the change in body weight was recorded at 24 h intervals (see Materials and Methods). The results in Table 1 show that LPS administration induces a transient but significant weight loss. Maximal change could be seen at 48 h after injection of 100 μg/mouse of LPS. Of note, maximal serum TNF and IL-6 levels in this system were found at 1–2 h and 3–4 h post-endotoxin (30 μg/mouse), respectively (data not shown). These results confirm those obtained previously.[11]

Inhibition of Weight Loss by Anti-IL-6 MAb

Next, we attempted to reverse the endotoxin-induced weight loss by the neutralizing anti-IL-6 antibody 20F3. Table 2 shows that pretreatment of mice with anti-IL-6 significantly reduced LPS-induced weight loss (10.9% in RIgG vs 3.2% in 20F3 treated mice at 24 h and 14.1% in RIgG vs 4.7% in 20F3 treated mice, 48 h post-endotoxin). Anti-TNF MAb reduced by *c.* 50% the LPS-induced weight loss, but only within 48 h.

Differential Involvement of IL-6 and TNF in LPS-Induced Metabolic Changes

Table 3 clearly demonstrates that pretreatment of mice with MAb anti-IL-6 significantly inhibits LPS-induced hypoglycemia. In the same set of experiments, the pretreatment of mice with anti-TNF MAb did not improve hypoglycemia. The same pattern of differential involvement of these cytokines, could be observed with regard to LPS induction of the hepatic acute phase reactant fibrinogen. Table 4 shows that whereas the anti-IL-6 MAb 20F3 significantly reduced plasma fibrinogen concentrations, anti-TNF antibody did not affect the level of this LPS-induced inflammatory marker. On the other hand, pretreatment with anti-TNF almost completely blocked the LPS-induced increase in serum triglycerides (Table 5), whereas the anti-IL-6 MAb failed to block LPS-induced hypertriglyceridemia.

Modulation of Serum IL-6 Levels by Anti-TNF and Anti-IL-6 MAb

To determine whether the anti-cytokine MAb used in this study affected LPS-induced IL-6 levels,

TABLE 1. Time and dose dependent responses of LPS-induced weight loss.

Treatment	% weight loss 24 h	48 h	72 h	96 h
PBS	0	−1.0 ± 0.5	−1.0 ± 0.5	2.0 ± 0.5
LPS (10 μg/mouse)	5.1 ± 1.3	2.1 ± 1.0	3.2 ± 1.0	−1.2 ± 0.5
LPS (30 μg/mouse)	8.2 ± 1.7	9.5 ± 2.5	5.3 ± 2.0	−1.8 ± 0.5
LPS (100 μg/mouse)	10.4 ± 0.9	16.2 ± 1.2	8.1 ± 0.8	2.1 ± 0.2
LPS (300 μg/mouse)	10.0 ± 1.1	16.1 ± 1.5	8.3 ± 0.8	NT

CD_2F_1 male mice received an i.p. injection of 0.25 ml of PBS or the indicated amount of LPS. Weight loss was determined as described in Materials and Methods on six mice per group. Results are expressed as mean ± SD of cumulative % weight loss. NT =

TABLE 2. Inhibition of LPS induces weight loss by anti-IL-6 MAb.

Group	Pretreatment	Treatment	% weight loss at 24 h	% weight loss at 48 h
1	PBS	PBS	0	0
2	PBS	LPS	12.2 ± 1.5	15.0 ± 1.7
3	RIgG	LPS	10.9 ± 1.6	14.1 ± 1.5
4	HIgG	LPS	11.6 ± 2.6	16.5 ± 1.6
5	20F3	LPS	3.2 ± 2.9*	4.7 ± 1.5*
6	TN3.19	LPS	10.6 ± 1.2	8.1 ± 1.0*

CD_2F_1 mice (six per group) were pretreated 16 h before LPS injection (100 μg) with 0.6 mg per mouse of RIgG and 20F3 or with 0.3 mg per mouse of HIgG and TN3.19. Percent weight loss between the time of LPS injection and 24 and 48 h was recorded. Results are expressed as mean ± SD. Asterisk represents a *P* value less than 0.01 of group 5 from 3 and 6 from 4.

mice were pretreated with antibody and at various time points after LPS injection, mice were bled and serum IL-6 levels were quantified by both the B-9 bioassay and an ELISA. Table 6 shows that pretreatment of mice with TN3.19 anti-TNF MAb reduced the amount of serum IL-6 by *c.* 50% as compared with the controls (PBS or HIgG). In contrast, pretreatment with anti-IL-6 MAb resulted in a tremendous increase in both bioactive and immunoreactive IL-6 levels as compared to the controls. At 4 and 6 h post-endotoxin, 20F3 increased the circulating IL-6 levels by 28- and 274-fold, respectively. Of note, these results were reproduced twice with similar results. Also, the addition of fresh 20F3 MAb to diluted serum sample from all the groups in the experiment abrogated the cellular proliferation of B-9 cells in the bioassay.

DISCUSSION

Pro-inflammatory cytokines, IL-1, TNF and IL-6 have been implicated in the pathophysiological events associated with septic shock, bacterial toxemia and a variety of chronic inflammatory conditions. This was based in part on experiments where the administration of recombinant cytokines could mimic most of the changes associated with inflammation. For example, injections of IL-1 and TNF induce fever, neutrophilia, hypotension, hepatic acute phase response, activate the hypothalamic–pituitary–adrenal axis, weight loss, hypertriglyceridemia, hypoglycemia as well as stimulate the induction of secondary mediators including IL-6. However, the relative contribution of each of these cytokines to the overall metabolic changes asso-

TABLE 3. IL-6 is involved in LPS-induced hypoglycemia.

Pretreatment	Treatment	Expt. 1	Expt. 2	Expt. 3	Expt. 4
PBS	PBS	148 ± 9	131 ± 8	130 ± 10	NT
PBS	LPS	86 ± 9	90 ± 3	NT	100 ± 5
RIgG	LPS	83 ± 5	89 ± 4	74 ± 3	96 ± 11
HIgG	LPS	81 ± 16	83 ± 5	NT	NT
20F3	LPS	115 ± 4*	106 ± 5*	112 ± 5*	123 ± 4
TN3.19	LPS	78 ± 3	82 ± 5	NT	NT
20F3	PBS	NT	NT	148 ± 10	NT
RIgG	PBS	NT	NT	126 ± 15	NT

CD_2F_1 male mice were pretreated as described in Table 2. In experiments 1, 3 and 4 the amount of LPS used was 30 μg/mouse. In experiment 2 the amount of LPS used was 100 μg/mouse. There were 4–5 mice per group. Mice were bled at 4 h post-LPS injection, except in experiment 4 where mice were bled at 6 h post-endotoxin. Results are expressed in mg/dl ± SD. NT = not tested.
* $P < 0.01$ from RIgG injected group.

TABLE 4. IL-6 is involved in LPS-induced elevation of fibrinogen.

Pretreatment	Treatment	Expt. 1	Expt. 2	Expt. 3
PBS	PBS	257 ± 15	239 ± 31	300 ± 61
PBS	LPS	568 ± 47	711 ± 160	523 ± 123
RIgG	LPS	619 ± 127	824 ± 49	454 ± 136
HIgG	LPS	693 ± 77	734 ± 48	554 ± 92
20F3	LPS	396 ± 5*	480 ± 78*	293 ± 22*
TN3.19	LPS	640 ± 61	860 ± 16	445 ± 76

See footnote to Table 3. Plasma was collected 24 h post-endotoxin. Results are expressed in mg/dl ± SD.
* $P < 0.01$ from RIgG-injected mice.

TABLE 5. TNF but not IL-6 is involved in LPS-induced hypertriglyceridemia.

Pretreatment	Treatment	Expt. 1	Expt. 2	Expt. 3
PBS	PBS	98 ± 10	112 ± 18	96 ± 10
PBS	LPS	171 ± 12	161 ± 28	170 ± 18
RIgG	LPS	NT	176 ± 33	180 ± 32
HIgG	LPS	178 ± 20	198 ± 25	169 ± 25
20F3	LPS	156 ± 17	163 ± 23	165 ± 8
TN3.19	LPS	116 ± 4*	106 ± 15*	100 ± 16*

See footnote to Table 3. Results are expressed in mg/dl ± SD. NT = not tested.
* $P < 0.01$ from HIgG-injected mice.

TABLE 6. Anti-TNF reduces and anti-IL-6 elevates circulating IL-6 levels.

Post-endotoxin assay		2 h	4 h		6 h
		B-9	ELISA	B-9	B-9
Pretreatment	Treatment				
PBS	LPS	NT	31 ± 10	33 ± 3	6 ± 3
RIgG	LPS	72 ± 10	32 ± 13	31 ± 10	7 ± 3
20F3	LPS	401 ± 50	975 ± 150	880 ± 100	1922 ± 300
HIgG	LPS	NT	NT	35 ± 7	NT
TN3.19	LPS	NT	17 ± 7	12 ± 3	NT

CD_2F_1 male mice (3–6 per group) were pretreated with PBS or antibodies as indicated in Table 2. At the indicated time points relative to LPS (30 μg/mouse) injection, mice were bled and serum samples were subjected to IL-6 bioassay (B-9) and ELISA. Results are expressed in units × 10^{-3}/ml or ng/ml for B-9 assay and ELISA, respectively. Normal levels of IL-6 in CD_2F_1 mice are 0.02 ± 0.01 ng/ml in ELISA. The addition of fresh 20F3 (10 μg/ml) abrogated cellular proliferation of B-9 cells in all samples tested. NT = not tested.

ciated with endogenously produced cytokines during inflammation is not fully appreciated.

Recently, we showed that IL-6 is involved in key parameters of wasting associated with the development of C-26 tumors in vivo. Thus, hypoglycemia, acute phase response and the loss of muscle and fat tissues were prevented by the rat anti-murine IL-6 MAb-20F3.[6] TNF did not appear to play a role in this experimental model, since anti-TNF MAb-TN3.19, did not improve cachexia. Therefore, it was of interest to determine how general is the role of IL-6 in inducing metabolic changes, especially in an experimental system where TNF is known to be produced. In this paper, we show that anti-IL-6 MAb inhibited LPS-induced weight loss, hypoglycemia and acute phase response. Interestingly, anti-TNF antibody only modestly reversed weight loss, did not improve the extent of hypoglycemia and acute phase response, but was very effective in blocking LPS-induced hypertriglyceridemia. Thus, these results appear to support our previous data obtained in the C-26 chronic inflammation model and to extend the role of IL-6 in mediating several metabolic alterations to an acute type inflammatory condition.

Chronic and acute infections have been associated with changes in glucose metabolism which could be reproduced by administration of IL-1, TNF but not IL-6. Typically, these changes involve an enhanced rate of glucose utilization in a variety of tissues, including liver, spleen, ileum and lung.[12] However, in septic animals or following TNF infusion, a corresponding increase in hepatic glucose production, probably related to elevation in glucagon levels,[13] partially compensates for the increase in glucose uptake resulting only in a modest hypoglycemia. Indeed, high doses of LPS used in our study reduced glucose levels by only 30 to 40%.

The finding that anti-IL-6, and not anti-TNF antibody, reverses significantly LPS-induced hypoglycemia confirms and extends previous findings which demonstrated that IL-1 receptor antagonist, but not anti-TNF antibody partially reverses LPS-induced hypoglycemia.[14,15] Whereas TNF itself, given in a sufficient dose (5–7.5 μg/mouse), induced a modest hypoglycemia, which was partially reversed by anti-IL-6 treatment,[16] the administration of IL-6 to mice did not lead to a reduction of blood glucose levels (our unpublished results). Surprisingly, anti-IL-6 antibody did not affect IL-1-induced hypoglycemia.[16] Taken together, these blocking experiments suggest that IL-6, like IL-1, contributes to LPS-induced hypoglycemia. However, unlike IL-1, which when given by itself induces hypoglycemia, IL-6 may require an interaction with other LPS-induced factors, the identity of which remains to be determined.

In contrast, our experiments with anti-TNF clearly show that the elevation in triglyceride levels—known to occur during infection[8]—involves TNF. These findings support previous results. Daily administration of TNF to animals induces hypertriglyceridemia,[17] which was linked to TNF-induced decrease in LPL activity.[3,4] More recent studies, however, suggested that TNF increases serum triglycerides by stimulating hepatic lipogenesis and not by inhibiting adipose tissue LPL activity or triglyceride clearance (for review see ref. 18).

Results presented here also show that the improvement in several LPS-induced inflammatory markers such as hypoglycemia, weight loss and acute phase response which involves IL-6 cannot be directly linked to a reduced serum level of this cytokine. Paradoxically, the anti-IL-6 20F3 induced a significant increase in serum IL-6 levels. This observation was recently made in 20F3-treated mice when the generalized Schwartzman reaction was induced. The 20F3 MAb provided partial but a significant protection against the reaction and yet resulted in an increase of circulating IL-6 levels.[19] Also, this phenomenon is not restricted to the 20F3 MAb, as anti-human IL-6 MAb prolonged serum IL-6 levels in mice injected

with recombinant human IL-6.[20] One explanation of this observation may involve an antibody-dependent slower clearance of the cytokine. However, the difficulty with this is that the increased level of IL-6 does not represent inactive antigen–antibody complexes. This is based on the following findings. First, the IL-6 in the circulation of anti-IL-6-treated and LPS-challenged animals is still active in the B-9 assay. Second, this activity in the bioassay was fully neutralized when fresh 20F3 was added. Third, a significant signal was obtained in an IL-6 specific enzyme-linked immunosorbent assay (ELISA) where the capturing antibody was 20F3. Together, this would argue that the increase in serum IL-6 may represent free cytokine in a form which is not bound to the antibody. A second explanation of this phenomenon is a potential feedback mechanism where the presence of the antibody in vivo induces excess synthesis of IL-6. However, it is difficult to assume that antibody-dependent increase in IL-6 level in vivo results in a protection against LPS-induced deleterious changes. IL-6 is currently designated as a pro-inflammatory cytokine, which is based in part on its ability to stimulate hepatic acute phase protein production[21] and pituitary hormones such as ACTH,[22] and to inhibit LPL activity in vivo.[7] Together with the demonstration that in our system, anti-IL-6 treatment greatly reduced fibrinogen levels, these data support the notion that the effect of IL-6 on the liver and possibly other tissues as well in LPS-treated mice, was neutralized. Furthermore, with a different assay system, no IL-6 could be found in the sera of IL-1 treated mice.[16] Thus, the significance of the increase in the B-9 assay and in the ELISA, in sera of mice receiving blocking antibody to IL-6, will require further investigation.

MATERIALS AND METHODS

Mice

Male Balb/C × DBA/2 (CD_2F_1) mice were purchased from Charles River Breeding Laboratories (Wilmington, MA), and were used at 10–12 weeks of age.

Reagents and Antibodies

LPS from *E. coli* 055:B5 was obtained from Sigma (St. Louis, MO). The rat IgG_1 anti-murine IL-6 MAb, 20F3 was originated by Dr J. Abrams (DNAX, Palo Alto, CA). For in-vivo studies, pharmaceutical-grade antibody was used (a gift from Dr C.O. Jacob, Syntex, Palo Alto, CA). The antibody was purified to > 98% by Prosep Protein A (Proton Products, Maidenhead, UK). The MAb TN3.19 was the kind gift of Dr R. Schreiber (Washington University, St. Louis, MO) and was purified on protein A agarose. Purified rat IgG (RIgG) and hamster IgG (HIgG) as control antibody for 20F3 and TN3.19 respectively, were purchased from Sigma.

Measurements of Glucose, Fibrinogen and Triglycerides

Mice were injected with antibodies intraperitoneally 16 h before LPS challenge as indicated in Tables 1–6. The quantity of 20F3 and RIgG was 0.6 mg/mouse and the quantity of TN3.19 and HIgG was 0.3 mg/mouse. Serum was collected for glucose and triglyceride determinations which were performed using an Ektachem DT-60 analyser (Eastman Kodak Co, Rochester, NY). Plasma fibrinogen was determined using a kit from Sigma (St. Louis, MO). Human fibrinogen was used as a standard.

Measurements of IL-6

IL-6 assays were performed as previously described.[23] One unit of IL-6 was defined as the amount required for half maximal stimulation of cell proliferation in the assay. The addition of 10 μg/ml of 20F3 abrogated proliferation of B-9 cells in response to diluted test samples. IL-6 was also quantified using a murine IL-6 ELISA from Endogen (Boston, MA). This ELISA uses 20F3 as a capturing antibody.

Statistical Analysis

Differences in weights and glucose, fibrinogen and triglyceride levels were compared by using computerized analysis of variance (ANOVA).

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